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Fungal endophyte effects on leaf chemistry alter the *in vitro* growth rates of leaf-cutting ants' fungal mutualist, *Leucocoprinus gongylophorus*

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ABSTRACT

Fungal symbionts that colonize leaf tissue asymptomatically (endophytes) can alter the foraging behaviour of leaf-cutting ants, and decrease the productivity of this herbivore's mutualistic fungal cultivar, *Leucocoprinus gongylophorus*. This negative effect of endophytes on the ant's cultivar could be the result of direct fungal–fungal interaction or indirect reductions in the quality of leaves, the cultivar's growth substratum. To test for the indirect effects, we measured *in vitro* growth rates of cultivars in media that contained sterilized leaf extracts from plants with high (E_{high}) and low (E_{low}) endophyte colonization. We found that, opposite to our expectations, cultivars grew significantly faster in E_{high} leaf extracts compared to E_{low} extracts. Our results suggest that endophyte-driven changes in leaf chemistry are a less likely explanation for the observed *in vivo* reduction in the ant's symbiotic fungal growth and imply that the effect of direct endophyte–cultivar interactions inside nests are potentially more important.

Introduction

Plant-associated microbial communities (plant microbiomes) have a substantial influence on a plant's ecological interactions and their responses to abiotic factors (Rodriguez et al., 2009; Porras-Alfaro and Bayman, 2011). An important part of this microbiome is a biologically and taxonomically hyperdiverse group of fungal symbionts that live asymptomatically inside plant tissues (hereafter 'endophytes' Wilson 1995). The ecological role of most endophytes has not yet been examined but studies so far suggest their effect on plant's ecological interactions is variable and range from negative to positive (Saikkonen et al., 2010). Certain plant-endophyte symbioses have been associated with reductions in infections by pathogenic fungi and with declines in herbivores' survival, developmental rates, fecundity and host acceptance (Webber, 1981; Clark et al., 1989; McGee, 2002; Jallow et al., 2004; Mejía et al., 2008; Van Bael et al., 2009; Crawford et al., 2010; Bittleston

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et al., 2011; Gange et al., 2012; Estrada et al., 2013). The protective benefits these fungi may confer on plants have motivated a large body of research focused on describing endophyte communities in a wide range of plants and in searching for species that target particular plant diseases (Suryanarayanan and Johnson, 2005; Arnold and Lutzoni, 2007; Helander et al., 2007; Mejia et al., 2009; Hanada et al., 2010; Higgins et al., 2011). However, in most cases the mechanisms underlying the effects that plant-endophyte interactions have on plant's natural enemies remain unclear, impeding both our understanding of the importance of endophytes in ecological networks and our evaluation of the potential to use them in biological control programs (Herre et al., 2007; Rodriguez Estrada et al., 2012).

Leaf endophytes have been associated with host preferences by leaf-cutting ants (Formicidae: Myrmicinae; *Atta*), a major herbivore in natural and agricultural ecosystems, primarily in the Neotropics (Van Bael et al., 2011). These ants can harvest more than 2% of leaf biomass in forest and savannas, play a strong role in shaping the species composition of plant communities, and are responsible for losses of up to a billion dollars yr^{-1} in agriculture (Abreu and Delabie, 1986; Hölldobler and Wilson, 1990; Wirth et al., 1997; Terborgh et al., 2001; Herz et al., 2007; Costa et al., 2008). Previous studies have shown that ants take longer to cut leaves with high relative to low loads of endophytes (Van Bael et al., 2009). In choice experiments when both types of leaves were present, recruitment of workers was higher to leaves with lower endophyte densities and these leaves were typically cut and removed faster than leaves with higher loads of the fungal symbionts (Bittleston et al., 2011; Estrada et al., 2013). The number of ants recruited to a resource indicates the quality of food (Jaffe and Howse, 1979) suggesting that endophyte colonization induces changes in leaves that lower their value to ants. Ant-cut leaf pieces are carried to subterranean nests and used to feed an obligate mutualistic fungal cultivar, *Leucocoprinus* (=*Leucoagaricus*) *gongylophorus* (Lepiotaceae, Basidiomycota) that ants and larvae use as their main source of nutrition. The fungal cultivar transforms leaf tissue into food for the ants that is delivered in specialized swollen hyphal tips called gongylidia (Cherrett et al., 1989; Aylward et al., 2012).

The presence of fungal endophytes in their forage material reduces the leaf-cutting ant's garden productivity in young laboratory colonies (Van Bael et al., 2012a). It is still unclear what mechanisms underlie this reduction or how those relate to the observed ant preferences for leaves with low loads of endophytes. The processing of leaf fragments by the ants greatly reduces the amount of alien microorganisms that enter the garden (Quinlan and Cherrett, 1977; Van Bael et al., 2009; Urriola et al., 2011). However, independently of the potential threat of a direct and detrimental interaction between the persisting endophytes and the ant's mutualistic fungi, endophyte symbioses with plants could indirectly affect garden productivity by reducing leaf quality. Endophyte colonization could result in leaf tissues that for the cultivar have lower nutritional value, less favourable pH or a higher content of antifungal metabolites than those from endophyte-free plants. This could be expected because the foliar content of some nutrients and minerals changes as a result of particular endophyte-plant symbioses (Rodriguez Costa Pinto et al., 2000; Gange et al., 2012; Estrada et al., 2013; but see Van Bael et al., 2012a). Furthermore, leaf chemistry also changes in ways detectable to ants (Estrada et al., 2013), which could be due to antifungal compounds expressed by plants in response to endophytes or toxins from these fungi that are constitutive or induced by plant defence responses (Schulz et al., 1999; White and Torres, 2010).

We conducted experiments to test the hypothesis that endophyte-driven changes in leaf extracts affect the leaf-cutting ants' fungal garden productivity. Our experiments removed the effects of leaf-cutting ant hygiene to observe *in vitro* growth responses of the fungal cultivar, *L. gongylophorus*. The ants' cultivar growth rate was compared in sterilized media containing extracts from leaves harvested from plants hosting low or high densities of endophytes. Since the sterilization process killed the endophytes in the leaves, our experiments focused on indirect rather than direct effects of the plant-endophyte symbiosis. Specifically, we tested for endophyte-mediated changes in leaf quality rather than a direct fungal–fungal interaction between endophytes and the ants' cultivar. We expected that the fungal cultivar would grow better in leaf extracts from plants with lower levels of endophyte colonization. We further assessed the growth rates of two leaf fungal symbionts in the same media to examine the extent to which patterns observed for the ants' cultivar extend to other fungi.

Materials and methods

Experimental plants

We made cuttings from a *Merremia umbellata* (Convolvulaceae) vine in Gamboa, Panama. These cuttings were planted individually in potting soil (Miracle-Gro, The Scotts Company LLC), and transferred to growth chambers kept at 28 °C, 85% humidity and with a 12 hr light:dark cycle. Inside the growth chambers we maintained vines so that new leaves were endophyte-free or with low rates of endophyte colonization by removing field-grown leaves and keeping the plant surfaces dry (Arnold and Herre, 2003). We chose pairs of plants with similar biomass to inoculate with the fungal endophyte *Colletotrichum tropicale* (Glomerellaceae, Ascomycota) (E_{high}), or to be kept as low endophyte controls (E_{low}). We inoculated them with a strain of *C. tropicale* originally isolated from leaves of *Cordia alliodora* (Boraginaceae) (strain Q633, GenBank accession no. GU994350), which has been commonly isolated from healthy leaves of several species of plants in Panama (Rojas et al., 2010). *Colletotrichum* species are ubiquitous in fungal endophytic communities (Arnold and Lutzoni, 2007; Rojas et al., 2010). Furthermore, bioassays with laboratory colonies of leaf-cutting ants have shown that ants take longer to cut *M. umbellata* leaves with greater percentages of colonization by *C. tropicale* compared to leaves with lower colonization (Van Bael et al., 2009).

To prepare experimental plants we sprayed conidia directly on leaves to produce E_{high} plants (Van Bael et al., 2009). The inoculation spray consisted of conidia (10^6–10^7 conidia ml^{-1}) suspended in sterile water and an emulsifying agent (Tween 20, Sigma–Aldrich, Inc.). Conidia

were obtained by liquid fermentation in 1.5 % Molasses Yeast Medium (15 g molasses, 2.5 g yeast extract, 1 l water). Then, to facilitate the colonization by the endophyte we kept plants in a high humidity environment inside a 0.5 × 1 m frame covered with clear plastic for 15 hr. Control plants selected to have a low density of endophytes (E_{low}) received the identical treatment as E_{high} plants except that the sprayed solution lacked conidia. After 15 hr we returned plants to growth chambers until they were used to make media for growth trials.

We assessed the density of endophytes in each E_{high} and E_{low} plant 7 d after the spray treatment. The test consisted of cutting a portion of the foliar lamina of one healthy leaf into 2 × 2 mm squares with a razor blade, sterilizing the surface of the leaf pieces by consecutive immersion in ethanol 70 % (1 min) and 10 % bleach (1 min), and plating sixteen of them on 2 % malt extract agar (MEA) plates. The effectiveness of this sterilization protocol killing all epiphytic microorganisms was verified by pressing sterile leaf pieces on MEA plates for a few minutes, removing the pieces, and monitoring the plates for microbial growth for at least 2 weeks. We estimated the density of endophyte colonization as the percentage of leaf pieces in a plate where *C. tropicale* growth was observed after 7 d of incubation at room temperature.

Fungal cultures

We cultured *L. gongylophorus* strains isolated from the fungal gardens of 13 *Atta colombica* colonies collected in Gamboa. Cultivars coexist with several microbial symbionts and contaminants in ants' gardens (Aylward et al., 2012). To obtain a pure culture of the fungal cultivar a few pieces of the middle portion of the garden were plated individually in Potato Dextrose Agar (PDA). Isolates in which only the cultivar grew were kept, transferred to new PDA plates, and incubated at room temperature (24 °C) for approximately 60 d before use in growth tests. We also performed growth tests with single strains of the leaf fungal symbiont *C. tropicale* and the apparently pathogen, *Cochliobolus* sp. (Pleosporaceae, Ascomycota). A strain of the latter species was isolated from tissue close to a necrotic lesion in wild *M. umbellata* and identified using molecular markers. In particular, ITS4 and ITS5 primers were used to amplify the nuclear ribosomal internal transcribed spacer region (ITS), the universal barcode marker for fungi (Schoch et al., 2012). The ITS sequence of the isolate (*Cochliobolus* sp. P001 GenBank accession no. KF840791) matched well with the sequence from strains in this genus (top match at 100 %, *Cochliobolus kusanoi* GenBank no. JX997754.1). We further examined the growth of the same *C. tropicale* strain used to inoculate plants. To prepare *Cochliobolus* sp. and *C. tropicale* for growth tests these fungi were subcultured and incubated at room temperature for 30 d in PDA or 7 d in 2 % MEA, respectively.

Growth tests

Three agar media were used to compare the growth rates of the ants' cultivar: water agar and water agar containing leaf extracts from E_{high} or E_{low} *M. umbellata*. Water agar contains low nutrition and thus mycelium growth in this medium was mostly fuelled by fungal reserves or nutrients from the original agar plug. This served as a control to compare the ants' cultivar growth in media containing leaves that may have included nutrients that support growth or contained growth-inhibiting antibiotic compounds.

To prepare media, healthy leaves from one to three plants were rinsed in running water and blended with deionized water in a 1:10 proportion of fresh weight to volume. The leaf suspension was then filtered and centrifuged (1700 *g*), mixed the supernatant liquid with more deionized water and agar (4 ml of water and 75 mg agar per each ml supernatant), and autoclaved it at 120 °C for 20 min (Arnold and Herre, 2003; Arnold et al., 2003; Bautista-Baños et al., 2003). We made three series of growth media, each one using a different set of E_{high} and E_{low} plants (hereafter "trial set"). In each of the three trial sets a subset of the 13 *L. gongylophorus* isolates were grown and in the second and third sets we grew replicates of the *Cochliobolus* sp. and *C. tropicale* strains. Leaves from all E_{high} plants used to make extracts had an estimated 100 % colonization of the endophyte while E_{low} leaves varied in their degree of colonization from 0 % in plants used in the first set, to 3 and 21 % in those for the second and third sets, respectively. Endophyte colonization for each experimental plant was estimated from one leaf with similar age and history as those used for extractions.

For trials, we subcultured each of the *L. gongylophorus* isolates onto five plates for each of the three media tested. The single strains of *Cochliobolus* sp. and *C. tropicale,* in turn, had 8 and 10 replicate plates per media, respectively. Subcultures started from 19.6 mm^2 plugs taken from the newer mycelium growth region of the fungal colonies and placed in the middle of the Petri dish. Plates were sealed and incubated in the dark at room temperature, maintaining them within the range of the optimal for *in vitro* cultures of Attini fungal symbionts (Powell and Stradling, 1986). The three fungi grow at relatively distinct rates, therefore, we measured mycelium growth after 2, 4 and 6 weeks for *L. gongylophorus,* 1–5 weeks for *Cochliobolus* sp. and 2–4 d for *C. tropicale*. We used the software ImageJ (NIH, USA) to measure mycelium area from digital photographs.

At the end of growth trials all plates were examined for the presence of gongylidia in the new growth of *L. gongylophorus* using a light microscope. Furthermore, the concentration of conidia produced in plates with *Cochliobolus* sp. and *C. tropicale*. was estimated. 1.5 ml of distillate water was added to each Petri dish and the agar surface softly scraped with a spatula. The suspension was then transferred to a clean vial and used to fill a hemacytometer. The number of spores was counted in a light microscope with 40× objective.

Statistical analysis

Mycelium growth areas were square-root transformed and growth rate was estimated as the slope of a linear regression for changes of area through time combining data for all replicates for a given *L. gongylophorus* isolate in each of the tested media (Baldrian and Gabriel, 2002). Linear regression fitted data well with R^2 ranging from 0.98 to 0.59 (median $R^2 = 0.93$). To examine the effect of the growth media on the growth rate of *L. gongylophorus* we used linear mixed-effects model with restricted maximum likelihood (REML) calculated with the R

package lme4 (Bates et al., 2013). This method was chosen over other linear models because experimental design was unbalanced and the isolates and plants used in each trial set could have affected mycelium growth (Bolker et al., 2009). We started with a model that contained growth media as a fixed effect and cultivar isolate number and trial set as random factors. Then, we searched for the optimal minimum model by modifying the structure of the random components and comparing the resulting models' parameters using F-statistics and Akaike Information Criteria (AIC) (Zuur et al., 2009). The selected model contained only the cultivar isolate as random factor. This model had the lowest AIC (114.7) and was not significantly different from the more complex model that also included the trial set as a random factor (AIC = 116.7, ANOVA, df = 1, $P = 1$). The selected model was further verified by a visual inspection of the random distribution of residuals and by a comparison with a null model only containing the random effects (AIC = 139, ANOVA, df = 2, $P < 0.001$). *P*-values were estimated with Markov-Chain Monte-Carlo sampling (MCMC) iterated 10^4 times and calculated using the R package LanguageR (Baayen, 2011). As *post hoc* tests, pairwise paired *t*-tests were performed with Bonferroni correction for the mean growth rate in the three growth media used (control, E_{low}, E_{high}).

Similar to *L. gongylophorus*, the growth rate of *Cochliobolus* sp. and *C. tropicale* was calculated for each medium by fitting a linear regression to the square-root transformed mycelium areas of all the replicates within the species. Because we had only one strain for each fungal species, growth rates were compared in the three growth media using the confidence intervals of the regression coefficient. Finally, the amount of conidia produced by the latter two fungi were compared in the three media using Kruskal Wallis' rank sum test, followed by pairwise Wilcoxon rank sum tests with Bonferroni corrections. Similar analyses were performed to examine the effect of the growth media on the final mycelium area of the three species of fungi. The results were comparable to those obtained for growth rate and are shown in the Supplementary Tables 1S and 2S. Statistical analyses were performed with the software package R (R Development Core Team, version 2.15.1).

Results

There was a significant effect of agar medium on *L. gongylophorus*' growth rate. Relative to the control (water agar), the leaf-cutting ants' mutualistic fungi grew about 1.5 times faster in plates with medium containing leaf extracts of E_{low} and about twice as fast in media with E_{high} *M. umbellata* leaf extracts (Table 1, Fig 1). The difference in growth rate between cultivars in E_{high} and E_{low} media was significant (paired *t*-test with Bonferroni correction, $P = 0.031$). No consistent difference was seen in the appearance of cultivar isolates between plates with E_{low} and E_{high} leaf extracts (Supplementary Fig 1S). The density of aerial mycelium and gongylidia clusters where comparable when plates where visually inspected or observed under a dissecting microscope, suggesting that 2-dimensional measures of the mycelium area covered by cultivars in leaf extract media are a good approximation of their growth rate.

Table 1 – Results of the linear mixed-effect model for growth rate of *L. gongylophorus*' mycelia with growth media as fixed effect and cultivar isolate as random factor[a]

Fixed effects	Estimate[b]	SE	t-value	pMCMC[c]
Intercept (Control)	2.496	0.276	9.058	
E_{low} growth medium	1.228	0.307	3.998	0.002
E_{high} growth medium	2.191	0.307	7.134	<0.001
Random effects		Variance		
Cultivar isolate		37.89 %		
Residuals		62.11 %		

a Number of observations = 39. Number of cultivar isolates (groups) = 13.
b Estimate given as the square root of the increase of mycelium area in a period of 14 days.
c *P*-values estimated with Markov-Chain Monte-Carlo sampling.

Nevertheless, in both leaf extract media, the aerial mycelium of cultivars was much more dense and typically contained more gongylidia clusters relative to colonies growing in control water agar. This implies that the observed differences in mycelium area underestimated the differences in the cultivars' growth rates between colonies growing in water medium and those using the leaf extracts media. The number of isolates that developed gongylidia in at least some of their peripheral hyphal tips was comparable among the growth media tested (chi-square test, df = 2, $P = 0.109$, 87 % in E_{high}, 81 % in E_{low} and 70 % in control).

The identity of the cultivar isolate contributed about 38 % of the random variation in the data (Table 1, Supplementary Fig 2S). This variation was in part due to the magnitude in the difference of growth rates between E_{high} and E_{low} media. For example, although 11 out of 13 isolates consistently grew faster in E_{high} compared with E_{low} leaf extracts, in only seven of them the slopes were significantly different (*i.e.* when the 95 % confidence intervals of E_{low} and E_{high} regression coefficients did not overlap). A linear mixed-effect model that

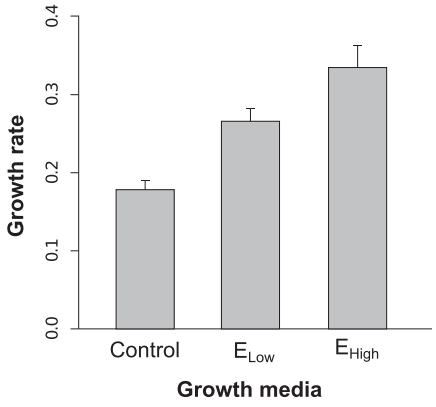

Fig 1 – Mean growth rate (square root mm^2 day^{-1} ± 1 SE) of the *Atta colombica*'s fungal symbiont *Leucocoprinus gongylophorus* in plates containing water agar (control) or water agar with leaf extracts of *Merremia umbellata* with low (E_{low}) and high (E_{high}) colonization of endophyte *Colletotrichum tropicale*.

included the trial set as random effect did not significantly improve the fit of the model to our data, showing that the particular leaf extracts used for making media contributed relatively little to the overall variation in growth rate. Nevertheless, the proportional increase in growth rate of cultivars in E_{high} media relative to E_{low} (E_{high}/E_{low}) was moderately correlated with the difference in the percentage of endophyte colonization in plants (Spearman's rank correlation $r = 0.59$).

In contrast to growth patterns observed for the leaf-cutting ant mutualistic fungi, the tested strain of the leaf symbionts, *Cochliobolus* sp. and *C. tropicale* grew at a similar rate and produced a comparable amount of conidia in E_{low} and E_{high} media plates (Table 2, Supplementary Fig 2S). The confidence intervals for the regression coefficient for mycelium area over time overlapped between samples cultivated in E_{low} and E_{high} but did not overlap between those and the controls (Table 2). Though *Cochliobolus* sp. and *C. tropicale* were grown in two trial sets, all samples of each species were pooled for analysis. The coefficients of determination were relatively high ($R^2 = 0.97$ and 0.91, max, min) except for control samples in *Cochliobolus* sp. ($R^2 = 0.59$). The growth rate of samples in control replicates for the second trial set was two times the growth rate for control replicates in the third trial (growth rates were 0.69 ± 0.12 and 0.31 ± 0.04, mean $\pm$ CI for second and third trial, respectively). Nevertheless, comparisons of the regression coefficient among media treatments within each trial set showed identical results as pooled data. The estimated concentration of conidia produced by both fungi was significantly different among media tested but this was due only to a major production in plates that contained leaf extracts (E_{high} and E_{low}) compared with plates with water agar alone (Table 2, Kruskal–Wallis tests, $X^2 = 6.01$, df = 2, $P = 0.049$ for *Cochliobolus* sp., and $X^2 = 15.37$, df = 2, $P < 0.01$ for *C. tropicale*).

Discussion

Our results do not support the prediction that changes in leaf extracts caused by endophyte colonization decrease leaf-cutting ants' fungal productivity. Contrary to our expectations, *L. gongylophorus* grew better in medium that contained sterilized leaf extracts from plants hosting high densities of endophytes than in any other agar media tested. Furthermore, endophyte colonization in *M. umbellata* did not affect growth or conidia production for the leaf symbionts, *Cochliobolus* sp. and *C. tropicale*, incubated in leaf extracts. The strength in the effect of media in cultivar growth was slightly associated with the difference in endophyte colonization between pairs of plants used to make leaf extracts. This result gives us confidence that growth responses found here were due to the effect of endophytes on leaf chemistry and not to unaccounted variation between plants unrelated to our treatments.

Table 2 – Estimated growth rate and conidia production of *M. umbellata* fungal symbionts growing in control (water) and leaf extracts from plants with high (E_{high}) and low (E_{low}) endophyte colonization

		Control	E_{low}	E_{high}
Cochliobolus sp.	Growth rate[1]	0.45 (0.11)^{A}	0.95 (0.05)^{B}	0.91 (0.06)^{B}
	Conidia[2]	0.75 ± 0.4^{a}	4.3 ± 2.8^{b}	2.49 ± 1^{b}
C. tropicale	Growth rate[1]	5.92 (0.41)^{A}	9.3 (0.99)^{B}	9.19 (0.88)^{B}
	Conidia[2]	0.72 ± 0.12^{a}	17.26 ± 4.27^{b}	12.7 ± 1.01^{b}

1 Slope of linear regression $\pm$ 95% confidence intervals (square root mm^2 d^{-1}) during 35 and 4 days of incubation for *Cochliobolus* sp. and *C. tropicale* cultures, respectively. Different letters show regression slopes that do not overlap.
2 Mean $\pm$ 1 SE of conidia ml^{-1} $\times$ 10^5. Different letters denote significant differences in mean concentration (pairwise Wilcoxon rank sum test with Bonferroni corrections).

Plants have a complex defence system against microbial infections (Jones and Dangl, 2006; Chisholm et al., 2006). The physical barrier of the cell wall and secondary compounds with antibiotic effect in cuticle and extracellular spaces are the first mechanism to halt hyphal grow. Besides these constitutive defences, fungal colonization induces several additional responses in plants that include the reinforcement of cell walls, accumulation of soluble sugars, production of oxygen active molecules (*e.g.* peroxides) and addition of new antibiotic compounds (phytoalexins) (Heath, 2000; Moerschbacher and Mendgen, 2000; Chisholm et al., 2006; Draper et al., 2011). At least part of the induced defence system seems to be activated during endophyte-plant interactions. Colonization of leaves by endophytes is known to cause the reinforcement of the cell walls in cells surrounding the point of fungal growth (Cabral et al., 1993; S. Maximova & E.A. Herre unpublished data). Furthermore, both plants and endophytes produce numerous antioxidants (*e.g.* phenolic compounds) presumably as the result of their antagonistic interaction (Schulz et al., 1999; Schulz and Boyle, 2005; White and Torres, 2010).

Extracts from *M. umbellata* leaves in our experiments likely contained water-soluble pectins, sugars, phenols, amino acids and minerals. Several phenolic compounds have been isolated from this plant (Yan et al., 2010). Growth patterns in the agar media tested suggest that these extracts provided nutrition to the developing fungi. Area covered by mycelium in E_{low} and E_{high} media increased by about two to sevenfold relative to area in water agar alone. Although both leaf extracts supported growth for the three fungi tested, E_{high} was a better medium for ants' cultivars but not for the two leaf symbionts. The cause for this difference in growth patterns remains unclear. Variation in cultivar growth rates in E_{low} and E_{high} media could result from a higher amount of nutrients in E_{high} agar media that this fungus but not the leaf symbionts could exploit. That would imply that endophytes metabolize cell wall polymers making them available to the cultivar or that plant and fungal defence compounds induced by the symbiotic interaction could be used by the cultivar as a source of nutrition. Alternatively, differences could originate from greater amounts of growth inhibiting compounds in E_{low} relative to E_{high} growth media for which leaf symbionts exhibit a higher degree of tolerance than the cultivar. That would imply that the endophyte *C. tropicale* could metabolize constitutive chemical defences in *M. umbellata*, decreasing the concentration of these compounds in E_{high} leaves compared to E_{low}. Detoxification of host defence compounds is one of the most common fungal tolerance mechanisms (Baldrian, 2006; Saunders et al., 2010). Species of *Colletotrichum*, in particular,

produce laccase enzymes that metabolize plant antifungal and structural phenols (*e.g* lignin), although the synthesis of these compounds is often associated with the strains' pathogenic activity and it is unknown to what extent they are also produced by endophytic species (Anderson and Nicholson, 1996; Guetsky et al., 2005; Baldrian, 2006).

Understanding the mechanisms of plant tissue decomposition by the ants' fungal garden has been the focus of extensive research (Aylward et al., 2012). Except for lignin, the fungal cultivar and a rich community of symbiotic bacteria associated with the garden have the capacity to metabolize all components of the plant cell walls, a process in which the host ants contribute substantially (D'Ettorre et al., 2002; Richard et al., 2005; De Fine Licht et al., 2010; Moller et al., 2011). Nevertheless, studies suggest that the main function of the decomposition of such wall polymers is to have access to the nutrients inside cells, where starch, proteins, phenols and soluble sugars are the main source of nutrition for the cultivar (Bucher et al., 2004; De Fine Licht et al., 2010). This implies that *L. gongylophorus* feeding characteristics and enzymatic abilities are close to those of typical fungal biotrophs like many leaf pathogens (Abril and Bucher, 2004; Schiøtt et al., 2010). Metaproteomic analysis and *in vitro* cultures of *L. gongylophorus*, however, show that this fungus produces the enzymes to break down cell wall polymers like xylan and pectin, and can successfully grow using them as source of carbon (Abril and Bucher, 2004; Silva et al., 2006; Aylward et al., 2013). Several endophytes can also fuel their growth using these polysaccharides *in vitro* (Petrini et al., 1992). Although this ability decreases or even disappears in several endophytes that only colonize leaves (Carroll and Petrini, 1983; Naffaa et al., 1998), certain species, including some *Colletotrichum*, synthesize cell wall polymer-degrading enzymes that allow them to transition to a saprobic lifestyle during leaf senescence (Promputtha et al., 2010). It is then an open question whether accumulation of cell wall components induced by the endophyte colonization or detoxification of plant antifungal compounds by endophytes could result in a richer food environment for the cultivar but not for the leaf symbionts in our growth media.

A previous *in vivo* experiment showed that cultivar colonization of leaf material after ants' preparation inside nests is independent of the abundance or diversity of endophytes that the substratum originally hosted (Van Bael et al., 2012b). This implies that changes in the chemistry of the leaves driven by endophytes do not disrupt the garden's *in vivo* growth although it does not test for the nutritional value of this substratum. The experiments reported here show that leaves that hosted these fungal symbionts are not a poorer substratum to support the cultivar growth than leaves never exposed to endophytes. If the ants' mutualist fungus grows similar, or even better, on foliar material that was colonized by endophytes, why do leaf-cutting ants prefer to harvest endophyte-free leaves? A possible explanation is that any benefit derived from a high quality of leaves is overridden by the threat that fungal–fungal interactions between endophytes and the ants' mutualist fungi will decrease the garden productivity. Endophytes that remain after substratum preparation can antagonize the fungal garden or remain in the garden as parasites (Fisher et al., 1996; Rodrigues et al., 2008, 2011). *L. gongylophorus* can inhibit endophyte growth when live strains of the endophytes and the cultivar are grown together *in vitro* (Van Bael et al., 2009). Nevertheless, if these fungal–fungal interactions also occur *in vivo*, presumably mediated by the production of antimicrobial compounds (Wang et al., 1999; Freinkman et al., 2009), they represent additional costs for the cultivar that may reduce garden productivity. The threat of negative effects from fungal–fungal interactions could explain why ants engage in elaborate and potentially costly cleaning behaviours when removing microorganism from leaves (Poulsen et al., 2002; Mangone and Currie, 2007; De Fine Licht and Boomsma, 2010; Griffiths and Hughes, 2010). In particular, leaf fragments hosting high loads of endophytes take about 40% longer to process than leaves without the fungal symbionts (Van Bael et al., 2012b). Alternatively, the pressure to reduce the chances of introducing specialized garden pathogens, which cause devastating effects on colonies (*e.g. Escovopsis*), might have selected for a generalized response of ants toward alien fungi (Currie et al., 1999; Pagnocca et al., 2012). Leaf-cutting ants were able to detect chemical changes in cucumber leaves hosting *C. tropicale* that influenced their foraging patterns between E_{high} and E_{low} leaves (Estrada et al., 2013). Whether these chemical compounds merely signal the presence of fungi to ants or indicate changes in leaf quality in this particular plant-fungal symbiosis is an open question.

Although our results clearly show that the changes in leaf extracts of endophyte-rich diet may not be a major factor influencing garden productivity, our approach has some limitations. First, studies have shown that sterilized medium containing aqueous leaf extracts retain antibiotic properties (Disalvo, 1974; Dutta et al., 1998; Maoz and Neeman, 1998; Arnold and Herre, 2003; Satish et al., 2007), but thermal degradation due to autoclaving could have altered the properties of some compounds in *M. umbellata* leaf extracts. This means that the advantages derived from E_{high} leaves observed in our *in vitro* cultures could not be reproduced *in vivo* if some components of the leaf chemistry that changed with sterilization had antibiotic effects. Second, our tests ignored the effect of endophyte-driven changes on leaf chemistry in the context of the whole microbial community that forms the ants' gardens. Such microorganisms play a key role in the digestion of plant material and in defence against garden pathogens (Aylward et al., 2012) and are known to change when colonies are fed with leaves rich in endophytes (Van Bael et al., 2012a). Finally, we inoculated *M. umbellata* leaves with one fungal strain but in natural conditions leaves typically host a rich and dynamic community of fungal endophytes (Herre et al., 2007). Endophyte species vary in the way they interact with plants and with other fungi (Cabral et al., 1993; Mejía et al., 2008; Saunders et al., 2010). Therefore, we cannot rule out that colonization by other fungi can trigger changes in leaf chemistry that negatively affect the cultivar's growth rate.

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Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funeco.2013.12.009.

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